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14. ABSTRACT

Our preliminary results indicate that the connective tissue growth factor (CTGF) is necessary and sufficient to block the oligodendrocyte maturation. The $Tsc1^{f/f}$;$SynCre^{+}$ mice show reduced myelination compared to wild type controls. We showed that by visualizing the oligodendrocytes by PLP promoter driven GFP expression. In addition to this finding, we now demonstrate the decrease in mature oligodendrocyte number *in vivo* by staining another mature oligodendrocyte marker, CC1. To determine the role of CTGF *in vivo*, we are in process of generating Tsc1;CTGF;SynCre+ mice. In order to investigate the effect of CTGF on oligodendrocyte maturation, we proposed to treat the oligodendrocytes with different domains of CTGF (Module I to IV). We are in process of generating HA-tagged versions of full length, and separate domains of CTGF. So far we cloned the full length, Module I and Module I and II of CTGF. In addition to the downstream effects of CTGF on oligodendrocytes, we are investigating the molecular mechanisms of upregulation of CTGF in TSC-deficient neurons. We focus on two main pathways, which were previously shown to regulate expression of CTGF.

15. SUBJECT TERMS
tuberous sclerosis, myelin, white matter, oligodendrocytes

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INTRODUCTION

Tuberous Sclerosis Complex (TSC) is an autosomal dominant syndrome characterized by many neurodevelopmental abnormalities. Vast majority of TSC patients develop neurological symptoms including epilepsy and autism spectrum disorders (Crino et al., 2006; Kwiatkowski et al., 2010; Tsai and Sahin, 2011). Past research has focused on formation of cortical tubers and epilepsy based on the theory that these abnormalities directly contribute to the cognitive deficits and seizure episodes. However, increasing evidence suggests a poor correlation between cortical tubers and the incidence of epilepsy or autism in TSC patients. Moreover, brains of TSC patients show disorganization of axon tracts and hypomyelination suggesting a role for the white matter in TSC neuropathology (Lewis et al., 2012; Peters et al., 2012). By generating a mouse model, we previously reported that Tsc1-deficiency in neurons alone is sufficient to give rise to hypomyelination as evidenced by reduced staining for myelin basic protein (MBP) (Meikle et al., 2008). Postnatal rapamycin treatment drastically improved MBP staining, suggesting that the myelination defect was dependent on neuronal mTOR activity; however, the underlying mechanism(s) remained unclear. In this study we investigate the hypomyelination in the neuronal *Tsc1*-knockout mouse and our preliminary results show that is due to arrested oligodendrocyte differentiation. Treating wild-type oligodendrocyte precursor cells (OPCs) with conditioned media from Tsc2-knockdown neurons was sufficient to mimic this phenomenon *in vitro*. By performing a genome-wide gene expression analysis, we identified connective tissue growth factor (CTGF) as a putative regulator of oligodendrocytes. We show that CTGF is upregulated in *Tsc2*-deficient neurons both *in vivo* and *in vitro.* Furthermore, CTGF is sufficient to inhibit the differentiation of the OPCs*,* and inhibition of CTGF in conditioned media from *Tsc2*-deficient neurons prevents the arrest of oligodendrocyte differentiation. Together, these studies provide the first mechanistic link between neuronal TSC1/2 function and oligodendrocyte maturation, thus myelination. While much of the pathology of TSC is established during embryonic development, myelination occurs predominantly in postnatal life. Therefore, improvement of myelination and thus neuronal connectivity could potentially be a therapeutic target in TSC.

In this study we focus on the mechanisms how CTGF regulates the oligodendrocyte maturation in detail.

BODY

Aim 1: To determine the role of CTGF in hypomyelination

A) Rescue of hypomyelination phenotype by knocking out CTGF in neurons lacking Tsc1 *in vivo*.

In order to investigate the effect of CTGF in the loss of TSC function, we started to generate mice, which lack both *Tsc1* and *CTGF* in neurons ($Tsc1^{f/f}$; $CTGF^{f/f}$; $SynCre^{+}$). We hypothesize that, as the loss of TSC1 in neurons results in an increase in expression of CTGF, and thus hypomyelination, the double knockout (missing both Tsc1 and CTGF) rescues this phenotype resulting in a similar degree of myelination as in wild type brains. We generated $Tsc1^{f/+}$;$CTGF^{f/+}$ mice and we are crossing these double heterozygous mutants with a $Tsc1^{f/f}$; $CTGF^{f/+}$; $SynCre^{+}$ and $Tsc1^{+/+}$; $CTGF^{f/+}$; $SynCre^{+}$ females with males lacking Cre in order to eliminate the germline recombination in the progenies. These crosses will provide us both the mutant ($Tsc1^{f/f}$; $CTGF^{f/f}$; $SynCre^{+}$) and the littermate controls ($Tsc1^{f/f}$; $CTGF^{+/+}$; $SynCre^{+}$ and $Tsc1^{+/+}$; $CTGF^{f/+}$; $SynCre^{+}$).

We will then stain the brains of the double mutant and the control mice with CTGF and different oligodendrocytes markers such as O4, MBP and CC1 to observe the changes in oligodendrocyte maturation, thus myelination. We will examine at least 6 brains for each. As a preliminary and a supporting experiment, we stained the wild type and $Tsc1^{f/f}$;$SynCre^{+}$;PLPGFP brains with CC1, to confirm the reduction in number of mature oligodendrocytes (Figure 1). These preliminary findings show that there is indeed a marked reduction in mature oligodendrocytes number in the Tsc1 mutants.

B) To test whether CTGF expression is altered in human TSC brain

We have so far stained perituber and tuber from 1 year old TSC patient brain and found that the CTGF expression is increased in cells, which have hyperactive mTOR, by staining for phosphoS6 (Figure 2). Most but not all phosphoS6 positive cells are stained for CTGF. These results strongly suggest that the observations we made in mouse brains also hold true for the human condition. We will perform additional staining this year with more samples.

C) Investigating the upstream pathways regulating the CTGF expression in Tsc-deficient neurons.

Our previous experiments show that the increase in the expression of CTGF in neurons lacking Tsc1/2 complex is a result of hyperactivation of mTOR pathway, which can be reversed by treatment with rapamycin, inhibitor of mTOR. Two previous reports on CTGF suggest that the expression of CTGF is increased by loss of serum response factor (SRF) and activation of Hippo pathway component, the transcription co-activator,

TAZ (Lai et al., 2011; Stritt et al., 2009). We therefore started to investigate the action of these two pathways in TSC-deficient neurons.

Our microarray data suggests that the mRNA levels of SRF are decreased in Tsc2-deficient neurons. In addition to its transcription activation, SRF also functions as repressor of transcription as shown for CTGF and Cyr61, another CCN family member. We validated the microarray data for SRF and some of its targets by quantitative real-time PCR (Figure 3A). In Tsc2-knockdown neurons, the transcript levels of SRF and its target Egr1 decrease whereas the Cyr61 transcript levels, which are suppressed by SRF, increase. Moreover, the protein levels of SRF show a reduction in Tsc2-knockdown neurons, which can be reversed by rapamycin treatment (Figure 3B). We will further investigate the role of SRF in CTGF expression in TSC2 deficient neurons by overexpressing SRF and analyzing the CTGF levels by western blotting.

In order to investigate the role of Hippo pathway in expression of CTGF in Tsc2-knockdown neurons, we analyzed the levels of YAP levels. Unphosphorylated form of YAP travels to nucleus and activates transcription of CTGF (Lai et al., 2011). We checked the mRNA and protein levels of YAP in Tsc2-knockdown neurons and found increased levels for both mRNA and protein in the Tsc2-knockdown (Figure 4). We will further investigate the role of YAP in expression of CTGF by knockdown of YAP by sh-RNAs in Tsc2-knockdown neurons and analyze the levels of CTGF by western blot.

Aim 2: To examine the mechanisms by which CTGF regulates oligodendrocyte differentiation.

In order to find out by which mechanism CTGF regulate the oligodendrocyte maturation, we proposed to dissect the effects of different CTGF modules. We initially investigated the effect of module IV on oligodendrocyte maturation since it is commercially available. Our preliminary experiment show that the treatment of oligodendrocytes by Module-IV results in an arrest in differentiation as observed by MBP staining (Figure 5). We will further investigate at which stage of differentiation CTGF affects by staining with stage-specific markers such as A2B5, PDGFRa, O1 and O4. In addition, we started to generate HA-tagged CTGF constructs to further dissect the roles of different modules. We have so far cloned full length, Module-I and Module-I&II. We are going to express these constructs in HEK293T cells and collect the media to treat oligodendrocytes.

KEY RESEARCH ACCOMPLISMENTS

The key research accomplishments during the first year of the grant:

-generation of mice double knockout for Tsc1 and CTGF

-identification of upstream regulators of CTGF expression

-demonstration of CTGF module-IV activity

- preliminary data on CTGF expression in human perituber and tuber

REPORTABLE OUTCOMES

Experiments are still in progress. There are no primary publications at this point. We plan to submit the first manuscript later in 2014.

We have published a review on tuberous sclerosis recently. In that review, we discuss white matter connectivity and acknowledge the funding support from the Department of Defense:

Julich K, Sahin M. Mechanism-based treatment in tuberous sclerosis complex. Pediatr Neurol 2013 Dec 5 pii: S0887-8994(13)00716-9. doi: 10.1016/j.pediatrneurol.2013.12.002. [Epub ahead of print]

CONCLUSIONS

We have made significant progress in both aims of the grant over the first year of funding. We plan on submitting the first manuscript describing our results later in 2014. In order to investigate the effect of CTGF on oligodendrocyte maturation *in vivo*, we have previously proposed to generate AAV2-CTGF-shRNA to reduce the expression of CTGF. Instead of this experiment, we chose to generate double knockout (Tsc1f/f, CTGFf/f, SynCre+) mice to observe a better physiological effect.

In addition, we now study the myelination of Tsc1 mutant brains by staining with a different mature oligodendrocyte marker, CC1, in addition to PLP promoter driven GFP.

Here we provide a preliminary data on expression of CTGF in human tuber and perituber sections. We will not only stain more sections from different patients for CTGF but also for different stage-specific oligodendrocyte markers such as O1, O4 and MBP.

Our preliminary findings on SRF and Hippo pathways suggest putative novel mechanisms between mTOR-SRF-Hippo Pathways. The crosstalk between these pathways will not only provide a better understanding of regulation of CTGF expression but also help us to understand the basic control mechanisms of cell growth and survival.

On the other hand, our preliminary data on the effect of CTGF on oligodendrocyte maturation so far provides information that Mod-IV is one of the domains of CTGF responsible for inhibiting oligodendrocyte maturation. Mod-IV was shown to bind to Wnt receptor (Mercurio et al., 2004). Moreover, Wnt pathway activation was shown to block the differentiation of oligodendrocytes (Feigenson et al., 2011). Therefore, we will investigate whether CTGF Mod-IV activate Wnt pathway in oligodendrocytes, which in turn block their maturation. In addition to Mod-IV, we will test the effects of other modules of CTGF on oligodendrocyte maturation in order to further analyze whether there is a mutual and/or complementary action of different modules.

REFERENCES:

Crino, P.B., Nathanson, K.L., and Henske, E.P. (2006). The tuberous sclerosis complex. N Engl J Med *355*, 1345-1356.

Feigenson, K., Reid, M., See, J., Crenshaw, I.E., and Grinspan, J.B. (2011). Canonical Wnt signalling requires the BMP pathway to inhibit oligodendrocyte maturation. ASN Neuro *3*, e00061.

Kwiatkowski, D.J., Whittemore, V.H., and Thiele, E.A. (2010). Tuberous sclerosis complex : genes, clinical features and therapeutics (Weinheim, Wiley-VCH).

Lai, D., Ho, K.C., Hao, Y., and Yang, X. (2011). Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. Cancer research *71*, 2728-2738.

Lewis, W.W., Sahin, M., Scherrer, B., Peters, J.M., Suarez, R.O., Vogel-Farley, V.K., Jeste, S.S., Gregas, M.C., Prabhu, S.P., Nelson, C.A., 3rd*, et al.* (2012). Impaired Language Pathways in Tuberous Sclerosis Complex Patients with Autism Spectrum Disorders. Cerebral cortex.

Meikle, L., Pollizzi, K., Egnor, A., Kramvis, I., Lane, H., Sahin, M., and Kwiatkowski, D.J. (2008). Response of a neuronal model of tuberous sclerosis to mammalian target of rapamycin (mTOR) inhibitors: effects on mTORC1 and Akt signaling lead to improved survival and function. J Neurosci *28*, 5422-5432.

Mercurio, S., Latinkic, B., Itasaki, N., Krumlauf, R., and Smith, J.C. (2004). Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. Development *131*, 2137-2147.

Peters, J.M., Sahin, M., Vogel-Farley, V.K., Jeste, S.S., Nelson, C.A., 3rd, Gregas, M.C., Prabhu, S.P., Scherrer, B., and Warfield, S.K. (2012). Loss of white matter microstructural integrity is associated with adverse neurological outcome in tuberous sclerosis complex. Acad Radiol *19*, 17-25.

Stritt, C., Stern, S., Harting, K., Manke, T., Sinske, D., Schwarz, H., Vingron, M., Nordheim, A., and Knoll, B. (2009). Paracrine control of oligodendrocyte differentiation by SRF-directed neuronal gene expression. Nature neuroscience *12*, 418-427.

Tsai, P., and Sahin, M. (2011). Mechanisms of neurocognitive dysfunction and therapeutic considerations in tuberous sclerosis complex. Curr Opin Neurol *24*, 106-113.

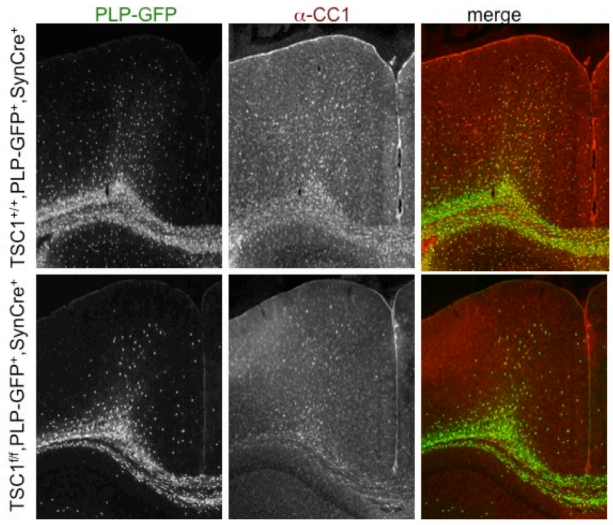

Figure 1. Staining of wild type and mutant mouse brains with CC1 (mature oligodendrocyte marker).

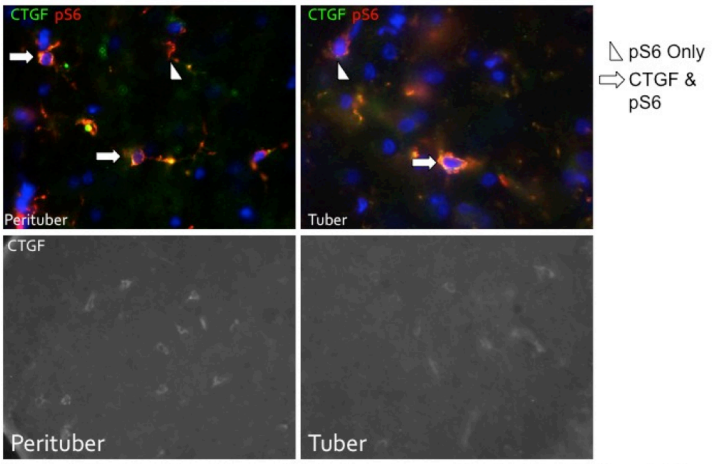

Figure 2. Staining of peritubér and tuber from 1 year old patient for CTGF and phoshoS6.

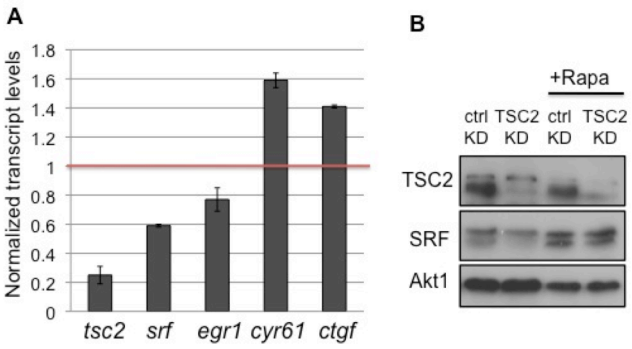

Figure 3. mRNA and protein levels of SRF decreases in TSC2 knockdown neurons. A) qPCR results of TSC2 knockdown cortical neurons. Data is normalized to control knockdown. B) SRF protein levels are decreased in TSC2 knockdown neurons, which can be reversed by rapamycin treatment.

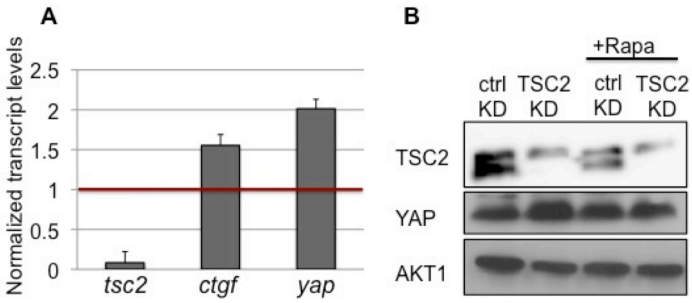

Figure 4. mRNA and protein levels of YAP increases in TSC2 knockdown neurons. A) qPCR results of TSC2 knockdown cortical neurons. Data is normalized to control knockdown. B) YAP protein levels are increased in TSC2 knockdown neurons, which can be reversed by rapamycin treatment.

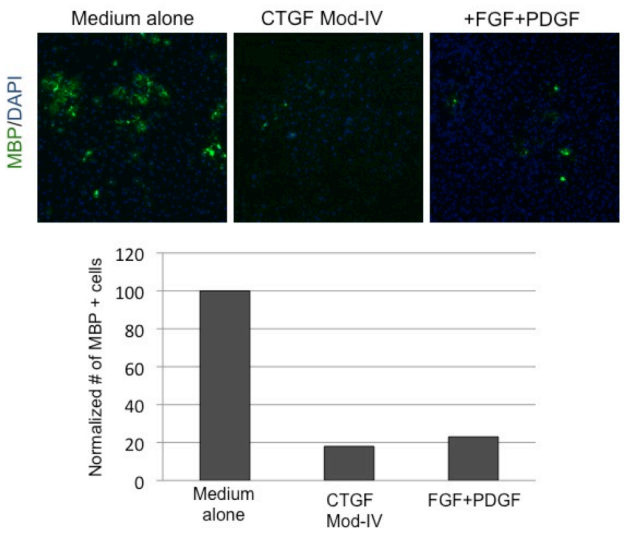

Figure 5. CTGF mod-IV blocks the maturation of oligodendrocytes. Oligodendrocytes treated with CTGF Mod-IV or FGF and PDGF (proliferating stage) for control. Oligodendrocytes are stained with MBP.